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(57) Claim

1. A method for producing at least one antibody which is not a monoclonal antibody against a pathogen; said method including the steps of:-

(a) providing a biological sample from an animal infected with, or challenged by, the pathogen or a pathogen preparation, said biological sample is taken from an infection site or an area of a lesion or an area close to or draining the infection site or lesion or leukocytes arising from the infection site and being found transiently detectable in lymph and blood.

(b) isolating cells from the biological sample;

(c) culturing said cells in vitro in a suitable culture medium; and

(d) harvesting antibodies produced from said cells.

5. A method according to any one of claims 1 to 4 wherein the cells isolated from the biological sample are B cells isolated at a time known to include a secretion- and/or an antibody- producing period.

~~In a preferred aspect, there is provided a method for preparing an antigen associated with a disease pathogen which method includes~~

~~providing~~

~~a sample of a disease pathogen taken from a disease pathogen at a stage of development during which it is thought to be most susceptible to attack; and~~

~~an antibody probe including at least one antibody against a disease pathogen produced by a method including~~

~~providing a biological sample from an immune animal taken a short time after the immune animal has been challenged with a pathogenic or pathogen extract;~~

~~isolating cells from the biological sample; culturing cells in vitro in a suitable culture medium; and~~

~~harvesting antibodies produced from said cells.~~

The disease pathogen from which a sample may be taken may be taken at a stage of development of the pathogen during which it is thought to be most susceptible to attack. For example, for a parasitic cestode infection, it may be suitable to take the sample from the oncosphere stage. It has been found that antigens present in the oncosphere stage of a parasitic infection are not present, for example, in the metacestode stage. For a parasitic worm infection, it may be suitable to take the sample from the larval, preferably late larval stage. For a parasitic fluke infection, it may be suitable to take the sample from the juvenile fluke stage.

The sample of disease pathogen may be mixed with a standard buffer solution and placed on a standard support such as an SDS-polyacrylamide gel to separate the proteins contained therein. The separated proteins may then be transferred to nitro-cellulose, nylon or other sheets.

The probing with a suitable antibody may further include subjecting the product produced thereby to a detection assay. The detection assay may include western blot techniques. The detection assay may be an



immunoprecipitation assay, a radioimmunoassay, an enzyme-linked immunoassay or immunofluorescent assay.

The at least one antibody produced as described above may be utilised simply in the form of the supernatant harvested from the culture medium. Alternatively, the antibodies may be separated and purified.

The antigen located as described above may be detected utilising any suitable assay technique.

In a further preferred aspect of the present invention the antibody contained in the culture medium may be used for the affinity purification, preferably immuno-affinity purification of antigen.

Accordingly in a preferred aspect there is provided a method for purifying antigen which method includes providing

a crude antigen mixture;

an antibody against a disease pathogen immobilised on a suitable support, which antibody is produced by a method including

providing a biological sample from an animal infected with, or challenged by, the pathogen or pathogen extract;

isolating cells from the biological sample;

culturing cells in vitro in a suitable culture medium; and

harvesting antibodies produced from said cells;

subjecting the crude antigen mixture to an affinity chromatography utilising the immobilised antibody; and

isolating the purified antigen so formed.

Antibody can be obtained from the culture supernatant probe by conventional methods. For example methods usually used to purify immunoglobulins from serum or plasma, e.g. precipitation with ammonium sulphate, fractionation with caprylic acid, ion exchange chromatography or by binding and elution from immobilised protein G or protein A may be utilised. Antibody so obtained can then be coupled to suitable supports, e.g. CNBr-activated Sepharose 4B (Pharmacia) Affi-gel (Bio-RAD) or other affinity chromatography supports able to bind proteins.

Immobilised antibody can then be applied to the

fractionation and purification of specific antigen from a complex parasite extract by affinity chromatography. After binding of antigen to immobilised antibody, unbound macromolecular species can be washed away from the solid support with, e.g. buffers containing 1.5M NaCl. Subsequently the antigen can be eluted from the affinity column with, e.g. low or high pH buffer or buffers containing chaotropic ions, e.g. 0.5 - 3.0 M sodium thiocyanate.

The application of the antibody probe to affinity chromatography enables sufficient quantities of specific antigens to be rapidly isolated from a complex crude extraction mixture for biochemical characterisation, amino-acid sequencing and vaccination of animals for limited protection studies. Application of affinity chromatography for obtaining antigen(s) avoids the difficulties often encountered when applying conventional biochemical techniques to the purification of an antigen about which little or no data is known. It also obviates the need to raise polyclonal or monoclonal antibodies for the purpose of "analytical" affinity chromatography. Large scale preparation may however require the preparation of polyclonal or monoclonal antibodies.

The antigens isolated or located may be used in the preparation of monoclonal antibodies. The monoclonal antibodies may form the basis of a passive treatment of the disease discussed above. Having identified the antigen(s) molecular biology or chemical techniques, e.g. cloning techniques may be used to produce unlimited amounts of this antigen or alternatively synthetic peptides corresponding to different fragments of the identified antigens may be used as a means to produce a vaccine.

Accordingly in a preferred aspect of the present invention there is provided a method for preparing a synthetic antigenic polypeptide against a disease pathogen, which method includes

providing

a cDNA library, or genomic library derived from a sample of a disease pathogen; and

an antibody probe selected from the group consisting of an antibody probe as described above;

a monoclonal antibody derived therefrom, or a derivative thereof;

generating synthetic polypeptides from the cDNA library or genomic library;

probing the synthetic polypeptides with the antibody probe; and

isolating the synthetic antigenic polypeptide detected thereby.

Either cDNA or genomic libraries may be used. The cDNA or genomic libraries may be assembled into suitable expression vectors that will enable transcription and the subsequent expression of the clone DNA, either in prokaryotic hosts (e.g. bacteria) or eukaryotic hosts (e.g. mammalian cells). The probes may preferably be selected from:

- (i) synthetic oligonucleotide probes based on the amino acid sequence of the antigen identified and purified as described above,
- (ii) antibodies obtained from the culture medium produced as described above;
- (iii) monoclonal or polyclonal antibodies produced against the antigens identified and purified as described above.
- (iv) recombinant or synthetic monoclonal antibodies or polypeptides with specificity for the antigen, e.g. as described by Ward et al 1989, Nature 241, pages 544 - 546.

Accordingly in a further aspect of the present invention, there is provided a protective antigen against a disease pathogen prepared by a method including

providing

a sample of a disease pathogen; and

an antibody probe including at least one antibody against a disease pathogen produced by a method including providing:

a biological sample from an animal infected with, or challenged by, the pathogen or pathogen extract;

isolating cells from the biological sample;

culturing cells in vitro in a suitable culture medium; and

harvesting antibodies produced from said cells.

The protective antigens may function as diagnostic antigens as discussed below.

Accordingly, in a preferred aspect of the present invention there is provided a protective antigen against Taenia hydatigena infections, selected from antigens having approximate molecular weights of 25 and 34 kilodaltons, as hereinafter described. As cross protection between various cestodes has been documented, similar antigens may also be detected in other cestode species, e.g. T. saginata, T. ovis, T. solium, Echinococcus granulosus.

In a further preferred aspect of the present invention there is provided a protective antigen against Haemonchus contortus infections, having an approximate molecular weight of 67 to 75 kilodaltons, as hereinafter described.

In a further preferred aspect of the present invention there is provided a protective antigen against Fasciola hepatica infections, having an approximate molecular weight of 120 to 125 kilodaltons, as hereinafter described.

In a still further preferred aspect of the present invention there is provided a protective antigen against Corynebacterium pseudotuberculosis infections, having an approximate molecular weight of 38 to 40 kilodaltons, as hereinafter described. This antigen is a protein antigen.

Accordingly, in a further aspect of the present invention there is provided a process for producing a monoclonal antibody against an antigen of a disease pathogen which method includes

providing

a B cell capable of producing antibodies against said antigen and obtained from an animal immunised with a protective antigen against the disease pathogen as described above; and

a myeloma cell;

fusing the B cell with the myeloma cell;

propagating a hybridoma formed thereby, and

harvesting the antibody produced by said hybridoma.

In a still further aspect the present invention provides a method for preventing diseases in animals, which



method includes administering to an animal an effective amount of at least one protective antigen prepared by a method as described above. Preferably the protective antigen is an antigen against a disease pathogen selected from the group consisting of Taenia hydatigena., Haemonchus contortus., Fasciola hepatica or Corynebacterium pseudotuberculosis as herein described.

In a still further aspect of the present invention there is provided a method for the treatment of diseases in animals, which method includes administering to an animal a therapeutically effective amount of a monoclonal antibody to a protective antigen produced as described above.

The present invention further provides a vaccine composition including a prophylactically effective amount of at least one protective antigen against a disease pathogen selected from the group consisting of Taenia hydatigena., Haemonchus contortus., Fasciola hepatica or Corynebacterium pseudotuberculosis as hereinafter described.

The present invention further provides a vaccine or veterinary composition including a therapeutically effective amount of at least one monoclonal antibody prepared as described above.

The vaccine or veterinary compositions according to the present invention may be administered orally or may be administered parenterally (for example by intramuscular, subcutaneous or intravenous injection) The amount required will vary with the antigenicity of the active ingredient and need only be an amount sufficient to induce an immune response typical of existing vaccines.

Reactive experimentation will easily establish the required amount. Typical initial doses of vaccine or veterinary compositions may be approximately 0.001-1 mg active ingredient/kg body weight. The dose rate may increase or multiple doses may be used as needed to provide the desired level of protection.

The vaccine or veterinary composition according to the present invention may further include a veterinary acceptable carrier, diluent or excipient therefor. Preferably the active ingredient may be suspended or dissolved in a carrier. The carrier may be any solid or

solvent that is non-toxic to the animal and compatible with the active ingredient. Suitable carriers include liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations, and solid carriers, such as talc or sucrose. Adjuvants, such as Freund's adjuvant, complete or incomplete, or immunomodulators such as cytokines may be added to enhance the antigenicity of the antigen if desired. When used for administering via the bronchial tubes, the vaccine is suitably present in the form of an aerosol.

In a still further aspect of the present invention there is provided a diagnostic kit including a diagnostic antigen against a disease pathogen identified and purified as described above.

The diagnostic kit may be utilised to detect infections in animals including Taenia hydatigena, Haemonchus contortus, Fasciola hepatica and Corynebacterium pseudotuberculosis.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the figures:

FIGURE 1A - H. contortus

Western blot from a 12.5% SDS polyacrylamide gel showing antigens in the L<sub>3</sub> and L<sub>4</sub> preparation (arrowed) identified by culture supernatant from immune-challenged sheep. Prestained molecular weight standards (BIORAD) are indicated.

FIGURE 1B - H. contortus

Western blot from a 7.5 to 15% gradient SDS-polyacrylamide gel showing antigens in the L<sub>4</sub> preparation (arrowed) identified by culture supernatant from immune-challenged sheep. Prestained molecular weights (Bio-Rad) are indicated.

FIGURE 2 - H. contortus

Silver-stained gel of affinity purified proteins on a 12.5% SDS-polyacrylamide gel under non-reducing

conditions. The affinity-purified proteins are in lane 1 and molecular weight standards (Pharmacia) are given ( $\text{mw} \times 10^3$ ). The region of antibody reactivity is bracketed.

FIGURE 3 - H. contortus

Western blot of affinity isolated antigens after probing with culture supernatant from a immune-challenged animal (Lane A). Prestained molecular weight markers (Bio-Rad) as in Figure 1.

FIGURE 4 - H. contortus

Western blot of affinity isolated antigen after incubating with Proteinase K (lanes A), trypsin (lanes B), Glycopeptidase F (lanes C) and control (no enzyme) (Lanes D). Molecular weights (Pharmacia) ( $\text{mw} \times 10^3$ ) are indicated.

FIGURE 5 - H. contortus

IEF capillary transfer to nitrocellulose from an agarose IEF gel ampholyte range 3 - 5 and probed with culture supernatant from an immune animal. Ph range using IEF standards (Bio-Rad) are given.

FIGURE 6 - F. hepatica

Western blot from a 7.5 - 15% SDS-PAGE gel.

MW = prestained molecular weight markers (BIORAD) as in Figure 1.

Lanes 1 & 4 - NEJ antigen preparation.

Lanes 2 & 5 - Adult fluke antigen preparation.

Lanes 3 & 6 - 12 day old fluke antigen preparation.

Lanes 1, 2 & 3 were probed with culture supernatant from HLN cells of infected cattle.

Lanes 4, 5 & 6 were probed with a mixture of culture supernatants from HLN of 10 day challenged sheep with an abbreviated or chronic primary infection.

Brackets = antigen claimed.

FIGURE 7 - F. hepatica

(A) 7.5 - 15% SDS-PAGE gel stained with silver nitrate.

lane 1 - high molecular weight markers (BIORAD) in reducing sample buffer.

lane 2 - prestained molecular weight markers (BIORAD).

lane 3 - Newly excysted juvenile (NEJ) antigen preparation in non-reducing

sample buffer.

Brackets = position of antigen claimed.

(B) 10% SDS-PAGE gel stained with silver nitrate.

lane 1: normal mouse serum in non-reducing buffer.

lane 2 & 3: n-butanol extracted antigen preparation in non-reducing sample buffer. Pellet (2) and supernatant (3) after 100,000 g spin for 50 minutes.

lane 4 & 5: Supernatant from NEJ sonicate. Pellet (4) and supernatant (5) after 100,000 g spin for 50 minutes.

Brackets = antigen claimed.

FIGURE 8 - F. hepatica

10% SDS gel stained with silver nitrate.

Bound (lane 2) and non-bound (lane 3) fraction after affinity purification of sonicated NEJ antigen preparation.

Brackets = position of antigen claimed.

Lane 1 = prestained molecular weight markers (BIORAD) as in Figure 1.

FIGURE 9 - T. nydatigena

Western blot from a 10% SDS-PAGE gel of oncosphere antigen in non-reducing sample buffer probed with culture supernatant from liver lymphocytes isolated from immune-challenged ( + ) or non-challenged ( - ) sheep.

FIGURE 10 - T. hydatigena

Probing of Western blots of oncosphere antigen (O) at dilutions of 1/2 and 1/4 and probing of Western blots of bladderwall metacystode antigen (B) preparations and scolex metacystode antigen (S) preparations with the positive culture supernatant as in Figure 9. 10% SDS-PAGE gel.

Arrows = antigens claimed

Sigma molecular weight markers run in reducing buffer.

FIGURE 11 - T. hydatigena

Probing of Western blots of oncosphere antigen with negative serum ( - ) or positive serum ( + ) taken from the same sheep at the same time after infection as the positive culture supernatant ( + ). 12% SDS-PAGE gel. Serum

dilution: 1/20.

FIGURE 12 - T.hydatigena

Western blot of oncosphere antigens probed with negative (1) or positive (2) serum or culture supernatant from leucocytes isolated from liver (3), hepatic lymph node (4) or prescapular lymph node (5) of recently infected animals. 10% SDS-PAGE gel.

FIGURE 13

Western blot on nitrocellulose from 12.5% SDS-PAGE - Samples solubilised in reducing buffer.

- A. Stained with amido black.  
Lane 1. Molecular weight standards.  
Lane 2. WA 1030 isolate cell extract.
- B. WA 1030 isolate cell extract probed with sera at 1:100 dilution obtained from a sheep infected with C.pseudotuberculosis.  
Lane 1. Serum taken immediately prior to infection.  
Lane 2. 2 days past infection  
Lane 3. 4 days past infection  
Lane 4. 7 days past infection  
Lane 5. 11 days past infection  
Lane 6. 14 days past infection

Arrows = protein antigen claimed."

FIGURE 14 - C. pseudotuberculosis

Isoelectric focusing of C. pseudotuberculosis antigen. Antigen has pH between 6.8 - 6.9.

EXAMPLE 1

Haemonchus contortus

Haemonchus contortus is an intestinal parasite of sheep that localises in the abomasum (fourth stomach). Late larval and adult stages of the parasite feed on whole blood. The parasite is responsible for sizeable economic loss to the sheep industry in Australia and considerable loss overseas and is a potentially fatal disease. Despite these losses no successful vaccine has been developed in the prior art against this parasite.

Parasite and experimental animals

H. contortus third stage larvae (L<sub>3</sub>) were collected from faecal cultures of donor sheep experimentally

infected with the parasite. Immune animals were obtained by repeatedly infecting sheep with H. contortus larvae and then monitoring faecal egg output. When a challenge dose produced no eggs in the faeces the animal was said to be immune. Once immune, the sheep were left for a period of at least four weeks before being challenged with 50,000 - 200,000 L<sub>3</sub> larvae and then killed five days post challenge.

#### Preparation of culture supernatants

Abomasal lymph nodes, draining the area of infection (abomasum) were removed and cell suspensions prepared as described for T. hydatigena below. Bulk cultures of 10 - 50 ml were set-up in culture flasks (Miles ) at concentration of  $2 - 4 \times 10^6$  cells/ml in culture medium. Preliminary experiments established that most of the antibodies in the culture supernatant were produced by the antibody secreting cells present in the in vivo stimulated lymph nodes and that this was not further increased by stimulation with pokeweed mitogen (PWM). PWM was therefore deleted from the cultures and culture supernatants were harvested after a five day incubation of cells at 37°C in a 5% CO<sub>2</sub> atmosphere and stored at -20°C until used.

#### Stage-specific recognition of antigens by culture supernatant

Third stage larvae of H. contortus were exsheathed in 0.05% sodium hypochlorite for 10 minutes at 37°C to remove the second stage sheath. The larvae were then repeatedly washed and centrifuged at 3,000 g for 10 minutes in phosphate buffered saline (PBS) pH 7.4. After the sixth wash they were transferred to 500 ml of DME medium pH 6.8 in the presence of 200 U/ml penicillin and 0.2 ug/ml streptomycin and cultured at 39°C with 20% CO<sub>2</sub> in air for 5 days. The culture media was then centrifuged at 3,000 g for 15 minutes at 20°C. L<sub>3</sub>, in vitro switched L<sub>4</sub> and adult parasites were removed and mechanically homogenised using a teflon pestle and a ground-glass tube for 20 minutes at 4°C in the presence of 0.1% Empigen zwitterionic detergent (Calbiochem) in PBS and 5mM phenylmethyl sulfonyl fluoride (PMSF). The homogenised larvae were immediately aliquoted and stored at -70°C. Frozen aliquots were thawed and mixed 1:1 with SDS non-reducing sample buffer and boiled for 5 minutes before being centrifuged for 2 minutes at 5,000

g and run on a 12.5% SDS-polyacrylamide mini gel (Bio-Rad) according to the method of Laemmli (1970). The gel was then electrophoretically transferred to nitrocellulose (Farlery de Rosbo et al, 1984) and probed with serum or culture supernatants from abomasal lymph node cells from immune challenged or non-challenged immune sheep. Western blots were blocked with 0.5% Tween 20 in PBS and all washes were done in 0.05% Tween 20 in PBS. The second antibody was a rabbit anti-sheep immunoglobulin coupled to horse-radish peroxidase (Dako) and then developed with 3,3'-diaminobenzidine (Sigma) and hydrogen peroxide.

Larval antigens were detected between approximately 67-75 kilodaltons (Kd) in the in vitro cultured fourth stage larvae (L<sub>4</sub>), and third stage (L<sub>3</sub>) exsheathed larvae of H. contortus when probed with culture supernatant from immune-challenged sheep (Figure 1). No antigens were identified at the molecular weights mentioned above for adult Haemonchus preparations (data now shown). There was no reaction when supernatant from non-challenged immune sheep was used to probe the blots (not shown), indicating that all the antibodies produced in the culture supernatant of the immune-challenged sheep were induced by the 5 day in vivo challenge. Similar probing of the Western blots with serum of the same animals taken at the same time reacted with several bands in all 3 parasite stages but did not highlight the 67 - 75 kD antigen (not shown). Also, as opposed to culture supernatant, no difference could be detected between serum of immune-challenged or immune non-challenged sheep.

#### Preparation of L<sub>4</sub> antigen for affinity purification

Crude antigen for affinity purification was prepared by shearing in vitro switched L<sub>4</sub> larvae with a polytron tissue homogeniser for 30 seconds on ice in the presence of 0.05% Empigen in 0.1 M Tris pH 8.0 and 5mM PMSF. After polytron treatment, SDS was added to a final concentration of 2% and the sample boiled for 3 minutes in a water bath, then centrifuged at 35,000 rpm for 30 minutes at 4°C. After centrifugation the SDS was removed from supernatant by precipitating the protein according to "Method A" described by Henderson, Oroszlan and Konigsberg (1979). After SDS removal, 7M Guanidine hydrochloride (IBI) was added to the

protein precipitate and allowed to stand for 5 hours on ice. An equal volume of 25% glycerol in distilled water was added and the entire sample dialysed against PBS and 0.05% Empigen pH 7.4. The sample was then applied to an affinity column.

#### Affinity purification of antigen

An affinity column was constructed to isolate the specific antigen by firstly removing antibodies from the culture supernatant of immune-challenged sheep using both Protein G Sepharose (Pharmacia/LKB) and donkey anti-sheep antibodies (Dako) coupled to Affi-prep (Bio-Rad) according to manufacturers specifications. The purified antibodies were then coupled to Affi-prep (Bio-Rad) according to the manufacturer's instructions and the column equilibrated in PBS and 0.05% Empigen detergent.

L<sub>4</sub> antigens were loaded onto the affinity column and unbound proteins removed using 0.5M NaCl pH 7.4 and 0.05% empigen. Bound proteins were eluted using 1M NH<sub>4</sub>SCN (Ajax) in PBS. The eluate was monitored by OD<sub>280</sub>. After elution the sample was extensively dialysed against 0.005M Tris pH 8.3, lyophilised and stored at -70°C. Samples were resuspended in distilled water and used for further analysis.

#### Silver-staining and Coomassie staining of affinity isolated antigen

On conventional SDS-Page gels several bands could be seen when silver staining the gels according to the method of Morrissey (1981) Figure 2. Immunoblotting and probing with culture supernatants from immune-challenged sheep resulted in a very intense antibody response in the 67- 75 kd region in the affinity isolated preparation (Figure 3). However no bands on either silver or coomassie stained gels could be positively correlated with this region of antibody reactivity. It would therefore appear that this particular molecule does not stain with the conventionally used protein stains.

#### Enzyme digests

6 ug of affinity isolated antigen from fourth stage larvae was incubated overnight at 37°C with either Glycopeptidase F (20 ul) (Boehringer Mannheim) or proteinase K (10 ug) (Sigma) in 0.1M Na-phosphate buffer pH 8.0 containing 10 mM EDTA, 0.1% SDS, 0.5% Triton-X-100 and 0.1%



$\beta$ -mercaptoethanol. In addition the antigen was incubated with trypsin (10 ug) (Difco ) in PBS. After incubation, all samples were mixed with SDS non-reducing buffer, boiled for 5 minutes and loaded onto a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with culture supernatant as previously described. Both proteinase K and trypsin treatment resulted in breakdown of the protein such that there was no recognition by antibodies in the culture supernatant (lanes A and B respectively). Glycopeptidase F had no effect on the antigen producing the same result as the control incubation (lanes C and D). This indicates that the antigen has a protein component which is degradable by proteinase K and trypsin, however the protein does not appear to contain asparagine-linked glycans under these conditions.

#### Iso-electric point of the antigen

Thin-layer iso-electric focussing (IEF) gels were prepared using a plastic template (Corning Immuno-electrophoresis plate) according to the method of McLachlan and Cornell (1978). Each IEF gel consisted of 0.95% w/v of IEF agarose (Bio-Rad), 11.4% w/v D-sorbitol (Sigma), 4.8% carrier ampholytes (Bio-Rad) range 3 - 5 or 3 - 10 and distilled water. The water, sorbitol and agarose were boiled, then placed on a 56°C water bath. The ampholytes were then added, the solution poured onto the template and a piece of Gelbond (FMC Pharmacia) overlayed. The template was placed in a plastic bag and stored at 4°C for at least 2 hours before use.

One  $\mu$ l of affinity purified antigen was applied and the gel was run at one watt constant power for 45 minutes. Upon completion of the run the gel was overlayed with nitrocellulose (Schleicher and Schuell) followed by several pieces of filter paper and a glass plate to act as a weight. Proteins then diffused from the gel to the membrane for one hour after which the membrane was blocked and probed with culture supernatant as previously described. The results indicated the presence of a highly acidic protein with an iso-electric point below 4.65 as indicated by IEF standards (Bio-Rad) Figure 5.

## EXAMPLE II

### Fasciola hepatica

Fasciola hepatica (liver fluke) is a parasite from the trematode family which can develop in the liver and bile ducts of many mammals and is of particular economic importance to the sheep and cattle industry. No prophylactic treatments such as vaccines against liver fluke are on the market. While sheep do not develop an immunity against F. hepatica, cattle can become partially resistant against reinfection. Although the mechanism of resistance in cattle has not been fully elucidated one possibility is that cattle recognise a different (protective) antigen than sheep. The technique described above was therefore used to study the differential antigen recognition between sheep and cattle.

#### Antigen Preparations

##### 1. Newly excysted juveniles (NEJ)

F. hepatica metacercariae (Mc) were purchased from Baldwin Aquatics (Monmouth, Oregon, U.S.A.). Metacercariae were excysted by suspension in 20 ml DME medium containing 100 mg Na-Tauro-cholic acid, 80 mg L-cysteine. The suspension was gassed in a mixture of 40% CO<sub>2</sub>, 10% O<sub>2</sub> and 50% N<sub>2</sub>, incubated at 37°C and NEJ collected after filtration through metal mesh. Sedimented NEJ were resuspended in phosphate buffered saline containing protease inhibitors (PBS-PI) (5mM Iodoacetamide and 1mM PMSF) and sonicated. Aliquots were stored at -70°C.

##### 2. Juvenile Flukes

Mice were infected orally with 30 Mc and killed 12 days later. Macerated livers were washed through a tea strainer and the juvenile flukes were recovered by differential centrifugation. Antigen was prepared as above.

##### 3. Adult Fluke

Infected livers from sheep or cattle were obtained from an abattoir. Adult flukes were recovered from the bile ducts, homogenised with a tissue grinder, aliquoted and frozen at -70°C.

#### SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

As for Haemonchus but only 7.5 - 15% gradient gels or 10% SDS-Page gels were run. All antigens were mixed 1:1

with non-reducing buffer. Bovine and ovine antibodies were detected using peroxidase-conjugated rabbit anti-bovine or sheep immunoglobulins (DAKO) respectively.

#### Butanol extraction

Newly excysted juveniles suspended in PBS-PI were sonicated and spun for 10 minutes at 5000 g. The supernatant was used for affinity purification as described later. The pellet was resuspended in PBS-PI and an equal volume of cold n-butanol was added. The mixture was incubated on ice for 10 minutes with occasional vortexing and then spun at 5000 g for 5 minutes. The water-soluble fraction was collected in the bottom layer.

#### Affinity purification

Antibodies were purified from the culture supernatant of infected cattle (see later) using sepharose bound protein G (Pharmacia) and used for the preparation of an affinity column essentially as described for H. contortus.

Triton X-100 R-S (Sigma) was added to a NEJ sonicate supernatant (see above) at a final concentration of 2%. The sonicate was loaded onto the affinity column and unbound fractions washed through with PBS containing 0.1% Triton X-100 R-S and 1 mM PMSF followed by 1.5 M NaCl. The bound fraction was eluted with 2 M  $\text{NH}_4\text{SCN}$  in PBS, dialysed against distilled water and freeze dried. Freeze-dried fractions were resuspended in PBS for further use.

#### Preparation of culture supernatant

Three sheep and three cattle were infected orally with 400 metacercariae. 18 days later they were dosed with FASINEX 120 (CIBA-GEIGY Australia Ltd.) to eliminate the primary infection. The animals were left for an additional 35 days before being challenged orally with 400 metacercariae. They were killed 10 days after challenge. Hepatic lymph nodes (HLN) were removed, cells isolated and incubated at  $2 - 4 \times 10^6$  cells/ml in culture medium with PWM as described for T. hydatigena and the supernatants harvested after 7 day in vitro culture. Additional culture supernatant was collected from HLN cells of sheep carrying an untreated primary F. hepatica infection (80 Mc) and challenged with 300 Mc 10 days before slaughter.

## Results

Probing of Western blots with culture supernatant from HLN cells of infected cattle resulted in strong antibody reactivity to a doublet antigen in the NEJ antigen preparation located slightly above the highest prestained molecular weight marker (Figure 6). Only one band at the higher molecular weight level of the NEJ doublet was consistently present in 12 day old fluke preparations while a similar band in the adult fluke preparation could not be reliably detected in several repeat experiments. In contrast, no reactions with NEI antigen were observed on Western blots when probed with culture supernatant of similarly infected (abbreviated primary and 10 day challenge) sheep (not shown). When the antigen preparations were probed with a mixture of this sheep culture supernatant and culture supernatant from chronically infected and challenged sheep, no reaction was observed with the band recognised by cattle supernatant although other bands were strongly recognised (Figure 6).

Unlike the restricted reactivity of the culture supernatant, similar Western blots probed with serum taken from the same animals at the same time reacted much more diffusely with a number of bands in all 3 parasite stages and no obvious difference between cattle and sheep serum could be observed (not shown).

Silver staining of the total NEJ preparation did not clearly stain the antigen recognised by the cattle culture supernatant suggesting it is a minor component of the total molecular make-up of the parasite (Figure 7a). By measuring the position of the antigen on a replicate Western blot relative to the highest prestained molecular weight marker, its approximate molecular weight on SDS-PAGE gel using BIORAD high MW markers as standards was calculated at 120 - 125 Kd. Clear silver staining of the doublet in the NEJ preparation could be seen when a n-butanol extracted soluble fraction of a NEJ sonicate pellet was run on an SDS-PAGE gel (Figure 7b).

Affinity purification on the bovine antibody column resulted in a clear depletion of the antigen from the non-bound fraction and high enrichment in the bound fraction (Figure 8). In addition there was also a strong low

molecular weight ( $\pm 24$  Kd) band in the bound fraction which also reacted strongly with the cattle culture supernatant on a Western blot (not shown) suggesting that the low MW band was due to breakdown of the 120 - 125 Kd antigen during the affinity procedure.

### EXAMPLE 3

#### Taenia Hydatigena

#### MATERIALS AND METHODS

##### Parasite and experimental animals

T.hydatigena eggs were collected from mature worm segments after purging of infected dogs with arecoline hydrobromide. 2 year old sheep kept on farms were used. At this age sheep have generally acquired immunity to T.hydatigena through natural exposure and this was confirmed in preliminary experiments. Positive sera and positive culture supernatants were collected from sheep killed 13 days after intraruminal injection of 40-50,000 T.hydatigena eggs. Negative culture supernatants were collected from sheep without prior challenge infection. Negative serum was collected from 5 month old sheep reared under wormfree conditions.

##### Preparation of leucocyte suspensions

Liver leucocytes were recovered by the following procedure. The sheep liver was removed and perfused via the portal vein with 1 litre of phosphate buffered saline (PBS) at room temperature followed by 0.5 liter of cold PBS with continuous and gentle massaging of the liver. This procedure resulted in complete blanching of the entire liver. Approximately 100 g of liver tissue was homogenized in a foodprocessor (Goldair, Australia) at low speed for 8-10 sec. The homogenized liver was then pushed through metal mesh, left to sediment for 8-10 min and filtered through cotton gauze. The cells were washed twice by centrifugation at 400 g for 8 min, followed by a low spin at 10 g for 5 min. to remove small clumps and the majority of hepatocytes. The supernatant of the last spin was collected and the remaining hepatocytes and dead cells removed by centrifugation over Ficoll/Isopaque gradients.

Leucocytes were also recovered from lymph nodes by cutting and teasing the nodes over a fine wire mesh. Dead

cells and clumps were removed by centrifugation over Ficoll.

#### Preparation of culture supernatant

Leucocytes were resuspended at a concentration of  $2-4 \times 10^6/\text{ml}$  in culture medium consisting of DME to which was added 10 mM HEPES, 100u/ml penicillin, 100 ug/ml streptomycin,  $2.5 \times 10^{-5}$  M 2-mercaptoethanol, 2 mM glutamine, 1 mM pyruvate and 10% fetal calf serum. Two ml cultures were set up in 24 well Linbro plates, stimulated with 25 ug/ml of pokeweed mitogen (PWM, Gibco Labs., Grand Island, NY) and incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. After 7 days the cultures were pooled and the cells sedimented by centrifugation. The supernatant was stored at  $-20^\circ\text{C}$  until used.

#### Preparation of antigens

##### (a) Oncosphere antigen (O)

T.hydatigena eggs recovered from mature worm segments were hatched with sodium hypochlorite and the released oncospheres were purified over 100% Percoll. A total of  $2.3 \times 10^5$  oncospheres were resuspended in 1 ml PBS containing protease inhibitors (PBS-PI) (5 mM Iodoacetamide and 1 mM phenyl methyl sulphanyl-fluoride) and frozen at  $-20^\circ\text{C}$ . This preparation was later thawed, sonicated with a MSE 150W ultrasonic disintegrator (Crawley, England), aliquoted and stored at  $-20^\circ\text{C}$ .

##### (b) Metacestode antigens

Metacestodes were collected from the peritoneal cavity of sheep at slaughter. The cyst fluid was removed, scolex and bladderwalls separated and frozen in PBS-PI at  $-20^\circ\text{C}$ . The preparations were later thawed, homogenised in a Kinematica Homogeniser (Polytron, Luzern, Switzerland), sonicated and centrifuged at 1400 rpm x 15 min. The supernatants were aliquoted and frozen at  $-20^\circ\text{C}$ .

#### Detection of antigens by the Western blotting technique

40 ul antigen fractions were mixed with 40 ul SDS non-reducing sample buffer, boiled for 5 min. centrifuged for 10 min at 5000 g and run on a 10 or 12% SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose sheets overnight. The sheets were blocked with 3% chicken ovalbumin (OVA) and cut into strips. Specific antigens on the strips were revealed after the

following incubation steps:

- (1) culture supernatant or serum,
- (2) biotinylated donkey anti-sheep Ig (Amersham)
- (3) streptavidin-biotinylated horseradish peroxidase complex (Amersham)
- (4) peroxidase substrate (0.6 mg/ml diaminobenzidine in PBS containing 0.05%  $H_2O_2$ ).

Reagents were used at manufacturers recommended dilutions.

- (1) Probing of Western blots of oncosphere antigen preparations revealed that there were 2 distinct antigen bands specifically recognised by culture supernatant collected from liver leucocytes of recently infected sheep ( + ) that were not recognised by culture supernatant collected from liver leucocytes of unchallenged sheep ( - ) (Figure 9, arrows).

These 2 antigen bands were still detected by the positive culture supernatant when the oncosphere preparation was diluted 1/2 and 1/4 and had approximate molecular weights of 22K and 35K (Figure 10).

- (2) When using the same positive liver culture supernatant to probe the Western blots of bladderwall (B) or scolex (S) preparations, the 2 oncosphere antigen bands were not detected (Figure 10). This indicates that the 2 antigen bands specifically detected in the oncosphere preparation are stage specific for the oncosphere, the parasite stage most likely to be susceptible to immune attack.

- (3) The 2 antigen bands detected when positive liver culture supernatant was used to "probe" the oncosphere Western blots were not detected when serum taken from the same sheep at the same time after infection was used as a probe (Figure 11).

- (4) The 2 oncospherical antigen bands were also detected when using culture supernatant from leucocytes isolated from the lymph nodes of recently infected animals (Figure 12).

#### EXAMPLE 4

##### Corynebacterium pseudotuberculosis

Caseous lymphadenitis (abbrev. CLA, also called Cheesy Gland) is a chronic infection of sheep and goats that is caused by the bacterium Corynebacterium pseudotuberculosis (syn. C. ovis). A complex cell-free vaccine for CLA (GLANVAC, Commonwealth Serum Laboratories) is known in the prior art and is currently administered either alone or as part of a 6 component antibacterial vaccine (6 in 1). The protection afforded by this vaccine is attributed to the inactivated toxin (i.e. toxoid) component. The toxin has a relative molecular weight of approximately 31 k daltons when run on 12.5% SDS-PAGE under reducing conditions. Whilst this prior art vaccine does generate some protective effect, the vaccine is complex and expensive, and significant numbers of infections may still occur.

This example describes the isolation of a protective antigen from Corynebacterium pseudotuberculosis infections, being approximate molecular weight of 38 - 40 kilodaltons.

##### Identification of Antigen

Culture supernatants were obtained using essentially the same methods as described for Haemonchus contortus (Example 1). Challenge infections of immune and naive sheep were localised to the lower leg by injection of a suspension of viable C.pseudotuberculosis and the popliteal lymph node was taken and set up in culture 5 to 7 days later.

Western blot analysis of whole cells using culture supernatants showed:

- (i) immune sheep recognised a complex pattern of antigens, but in the majority of animals the 38 - 40 kilodalton antigen was immunodominant
- (ii) naive (non-immune) animals strongly recognised the 38 - 40 kilodalton antigen and usually showed little other reactivity.

##### Extraction of antigen

Confluent growth of C.pseudotuberculosis is obtained on Brain Heart Infusion Agar by aerobic incubation at 37°C for between 1 and 3 days. Cells are obtained by scraping from the solid medium.

The bacterial mass is resuspended in sterile water



and washed by vortexing, then centrifugated at 3000 Xg for 15 mins. The supernatant is decanted and the wet cell pellet extracted by resuspension in approximately 4 times its volume of a solution of 1.0% (w/v) sodium dodecyl sulphate (SDS) in water and heating to between 70°C - 100°C for 10 minutes. Cells and debris are removed by centrifugation at 10,000 Xg. for 15 minutes. The crude mixture contains antigens other than that claimed.

The protein antigen can be purified from the complex mixture extracted with SDS from whole cells of C. pseudotuberculosis, as described above, or from cell-free culture supernatant. Conventional biochemical techniques can be used including ion exchange and molecular-sieve chromatography, ammonium sulphate fractionation together with hydrophobic chromatography. In addition, affinity chromatography can be used employing antibody isolated from the probe, immobilised on a suitable solid phase. Alternatively, polyclonal or monoclonal antibodies can be raised by immunisation of animals with purified antigen.

The antigen remains soluble in ammonium sulphate solutions up to 50% saturation.

#### Characterisation of 38 - 40 kD antigen.

The protein has an apparent molecular weight of between 38 - 40 kilodaltons when run under reducing conditions on a 12.5% SDS-polyacrylamide gel. It can be stained with Coomassie brilliant blue R250 and by silver stain. An amido black stain on nitrocellulose after Western blotting may also be used. Results are illustrated in Figure 13A.

Thin layer isoelectric focusing in 0.95% agarose containing 11.4% sorbitol and pH 5 - 8 ampholines localises the antigen to a pH 6.8 - 6.9 using the following Biorad IEF markers (see Figure 14): phycocyanin - 4.65, B-lactoglobulin B-5.10, bovine carbonic anhydrase 6.0, human carbonic anhydrase 6.5, equine myoglobin 7.0, human hemoglobin A 7.1, human hemoglobin C 7.5, cytochrome C 9.6.

Amino-acid sequence analysis of the native antigen that had been eluted from a SDS-polyacrylamide gel reveals a portion of the sequence at or near the amino terminus to be (progressing toward the carboxy terminus):

E S A T L S K E P L K A S P G R A D T, V G V Q  
(or if preferred Glu, Ser, Ala, Thr, Teu, Ser, Lys, Glu, Pro,  
Leu, Lys, Ala, Ser, Pro, Gly, Arg, Ala, Asp, Thr, Val, Gly,  
Val, Gln.

~~Finally, it is to be understood that various other~~  
modifications and/or alterations may be made without  
departing from the spirit of the present invention as  
outlined herein.

#### References

- Henderson, L.E., Oroszlan, S. and Konigsberg, W.  
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~~and Winter, G. 1989, Nature 341: 544-546.~~



#### Example 5

In vivo demonstration of the efficacy of 40Kd antigen as a vaccine antigen for caseous lymphadenitis (CLA).

A vaccine field trial was performed in 6 month old CLA free sheep which had not been previously vaccinated.

Antigen was prepared from the cell-free supernatant of a culture of C.pseudotuberculosis. The vaccine preparation was composed of approximately 40% 40Kd antigen by Coomassie staining of SDS-PAGE. The only band reacting on a Western blot probed with immune sheep serum was the 40Kd antigen, the other non-reacting substances being non-antigenic small molecular weight ( $< 20\text{Kd}$ ) peptides originating from culture medium components and high molecular weight material that stained red-brown with silver stain and was assumed to be lipid.

The sheep were immunised twice at four week intervals with either  $5\mu\text{g}$  or  $100\mu\text{g}$  of 40KD antigen per dose; aluminium hydroxide was used as adjuvant. The vaccinated sheep and unvaccinated control animals were challenged 4 weeks following the second booster immunisation by subcutaneous injection of a washed suspension of viable C.pseudotuberculosis cells on the lower part of the back left leg.

All animals were slaughtered at an abattoir in random order and the carcasses scored blind for CLA lesions by meat inspectors.



VACCINE GROUP  (No. in Group)	LESION SCORE		
	POPLITEAL LYMPH NODE LESIONS  (No./GRP)	LUNG LESIONS	
		No. GRP	Mean No./SHEEP
5 g 40Kd Antigen (22)	0	9	1.4
100 g 40Kd Antigen (21)	0	2	0.1
Unvaccinated Controls (22)	5	9	6.7

The 40Kd antigen confers significant protection against challenge with C. pseudotuberculosis.

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Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

#### References

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for producing at least one antibody which is not a monoclonal antibody against a pathogen; said method including the steps of:-
- (a) providing a biological sample from an animal infected with, or challenged by, the pathogen or a pathogen preparation, said biological sample is taken from an infection site or an area of a lesion or an area close to or draining the infection site or lesion or leukocytes arising from the infection site and being found transiently detectable in lymph and blood.
- (b) isolating cells from the biological sample;
- (c) culturing said cells in vitro in a suitable culture medium; and
- (d) harvesting antibodies produced from said cells.
2. A method according to claim 1 wherein the biological sample is taken from an immune animal.
3. A method according to claim 2 wherein the biological sample is taken within a period that the pathogen is vulnerable to immune attack after the immune animal has been challenged with a pathogen or pathogen preparation.
4. A method according to any one of claims 1 to 3 wherein the disease pathogen is selected from the group consisting of parasites, tumours, bacteria, viruses, mycoplasmas, chlamydias, rickettsias, spirochetes, fungi, protozoa, helminths, and ectoparasitic arthropods, or is an autoantigen.
5. A method according to any one of claims 1 to 4 wherein the cells isolated from the biological sample are B cells isolated at a time known to include a secretion- and/or an antibody- producing period.
6. A method according to claim 5 wherein the B cells



are isolated within a time period that the parasite or pathogen is vulnerable to immune attack after the immune animal has been challenged with a pathogen or pathogen preparation.

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7. A method according to any one of claims 1 to 6 wherein the cells isolated from the biological sample include antibody forming cells and memory B-cells.

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8. A method according to any one of claims 1 to 7 further including a step of adding a cell activating agent to the culture medium to activate and/or proliferate the cells isolated to secrete and/or release antibodies.

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9. A method according to claim 8 wherein the cell activating agent is selected from the group consisting of mitogens and helper factors, or their synthetic equivalents, pathogen derived molecules or a combination thereof.

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10. An antibody probe including at least one antibody which is not a monoclonal antibody against a disease pathogen said antibody produced by the method according to any one of claims 1 to 9.

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11. A method for identifying antigens; said method including:

(a) providing a crude antigen mixture;

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(b) providing at least one antibody which is not a monoclonal antibody against a pathogen; said antibody produced by the method according to any one of claims 1 to 9;

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(c) probing the crude antigen mixture with at least one antibody to identify at least one antigen recognized by the antibody.

12. A method for purifying an antigen; said method including:



(a) providing a crude antigen mixture;

(b) providing an antibody which is not a monoclonal antibody against a pathogen immobilized on a suitable support; said antibody produced by a method according to any one of claims 1 to 9.

(c) subjecting the crude antigen mixture to affinity chromatography utilizing the immobilized antibody; and

(d) isolating the purified antigen.

13. A method for preparing an antigen associated with a pathogen; said method including:

(a) providing a sample of pathogen;

(b) providing an antibody probe including at least one antibody which is not a monoclonal antibody against a pathogen said antibody produced by a method according to any one of claims 1 to 9;

(c) probing the pathogen with the antibody probe to detect at least one antigen; and

(d) isolating the antigen detected.

14. A method according to claim 13 wherein the sample of the pathogen is selected from the group consisting of parasites, tumours, viruses, chlamydias, rickettsias, mycoplasmas, bacteria, spirochetes, fungi, protozoa, helminths, ectoparasitic arthropods or where the antigen is associated with an autoimmune disease.

15. A method for preparing an antigen associated with a pathogen; said method including:

(a) providing a sample of a disease pathogen taken from a diseased animal at a stage of development during which it is thought to be most susceptible to attack;

(b) providing an antibody probe including at least one antibody which is not a monoclonal antibody against a pathogen said antibody produced by a method according to any one of claims 1 to 9;

(c) probing the pathogen sample with the antibody





antibody; and  
(d) isolating the antigen detected.

- 5 16. A method according to claim 15 wherein the disease pathogen is a parasitic cestode infection and the sample is taken from the oncosphere stage.
- 10 17. A method according to claim 15 wherein the disease pathogen is a parasitic worm infection and the sample is taken from the larval stage.
- 15 18. A method according to claim 15 wherein the disease pathogen is a parasitic fluke infection and the sample is taken from the juvenile fluke stage.
19. An antigen prepared according to the method of any one of claims 13 to 18.
- 20 20. A protective antigen against Taenia hydatigena infections prepared according to claim 13, selected from antigens having approximate molecular weights of 25 and 34 kilodaltons, as hereinbefore described.
- 25 21. A protective antigen against Haemonchus contortus infections prepared according to claim 13, having an approximate molecular weight of 67 to 75 kilodaltons, as hereinbefore described.
- 30 22. A protective antigen against Fasciola hepatica infections prepared according to claim 13, having an approximate molecular weight of 120 to 125 kilodaltons, as hereinbefore described.
- 35 23. A protective antigen against Corynebacterium pseudotuberculosis infections prepared according to claim 13, having an approximate molecular weight of 38 to 40 kilodaltons, as hereinbefore described.

24. A protective antigen according to claim 23 that provides protection for an animal against Corynebacterium pseudotuberculosis infections having a molecular weight of 40 kilodaltons and having an NH<sub>2</sub> terminal sequence of Glu, Ser, Ala, Thr, Leu, Ser, Lys, Glu, Pro, Leu, Lys, Ala, Ser, Pro, Gly, Arg, Ala, Asp, Thr, Val, Gly, Val, Gln.

25. A pharmaceutical composition including an antibody prepared to an antigen according to any one of claims 19 to 24 in a pharmaceutically acceptable carrier.

26. A diagnostic kit including an antigen according to any one of claims 19 to 24.

27. A diagnostic kit including an antibody prepared according to claim 1.

28. A method of treatment of disease in a patient in need; said method including a step of administering to said patient an amount of an antibody prepared to an antigen according to claim 19 therapeutically effective for the disease being treated.

29. A method according to claim 1 substantially as hereinbefore described with reference to the Examples.

30. A method according to claim 13 substantially as hereinbefore described with reference to the examples.

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